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# On the blockade of acetylcholine release at mouse motor nerve terminals by β-bungarotoxin and crotoxin

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**1** β-Bungarotoxin and crotoxin are phospholipose $A_2$ neurotoxins, which block irreversibly the evoked release of acetylcholine from motor nerve terminals of mouse triangularis sterni preparations.

**2** Extracellular recording of nerve terminal action potentials reveal that inhibition of transmitter release is not associated with failure of the action potential to invade nerve terminals.

**3** When evoked transmitter release (measured as intracellularly recorded endplate potentials) was blocked by β-bungarotoxin, spontaneous acetylcholine release was stimulated as in control experiments by $K^+$-induced depolarization and by the $Ca^{2+}$-ionophore A23187.

**4** The site of action of the toxins remains to be elucidated but would appear to be associated with the coupling of action potential induced-depolarization to the release mechanism, rather than with the release mechanism itself.

## Introduction

Neurotoxins from snake venoms with phospholipase $A_2$ activity (e.g. β-bungarotoxin, crotoxin, taipoxin) cause muscle paralysis by blocking acetylcholine release (see Chang, 1985; Harris, 1985 for reviews). The mechanisms responsible are unknown, but several have been proposed. These include depletion of energy stores, inhibition of choline uptake, excessive accumulation of $Ca^{2+}$, a decrease in $Ca^{2+}$ influx and a decrease in the efficacy of $Ca^{2+}$ in promoting release (see Chang, 1985). Some of these suggestions could be tested if the ionic currents controlling acetycholine release could be monitored. The small size of nerve terminals of mammalian neuromuscular junctions makes intracellular recording impossible but the local electrical activity of nerve terminals can be recorded with extracellular electrodes. Contributions related to $Na^+$, $K^+$ and $Ca^{2+}$ currents can be distinguished (Mallart, 1985; Penner & Dreyer, 1986). Using this technique, we found that the initial phase of facilitation of acetylcholine release, which is characteristic of the actions of β-bungarotoxin and crotoxin at mouse neuromuscular junctions, is associated with a reduction in terminal $K^+$ currents (Rowan & Harvey, 1988). We have now tested these two toxins under conditions in which transmitter release is blocked to determine if the toxins block the invasion of the action potential into the nerve terminal or the $Ca^{2+}$ current of the terminal. We have also examined whether the release mechanisms may be affected by the toxins by testing for effects of β-bungarotoxin on the stimulation of release by $K^+$-induced depolarization and by the $Ca^{2+}$ ionophore A23187.

## Methods

### *Nerve-muscle preparation*

Experiments were performed on the mouse triangularis sterni nerve-muscle preparation, as described previously (Rowan & Harvey, 1988). Preparations were perfused at a rate of 5–10 ml min$^{-1}$ with physiological solution (composition, mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, glucose 11.1 and $NaHCO_3$ 25 to buffer at pH 7.3).

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### *Studies on evoked release and on nerve terminal action potentials*

The intercostal nerves were stimulated every 2 s with pulses of 50 μs duration and supramaximal voltage. The potential difference between a silver/silver chloride reference electrode in the bath and the recording microelectrode (containing 3 M KCl, resistance 5-10 MΩ) was recorded as previously (Rowan & Harvey, 1988). In preparations paralysed by tubocurarine (1–2 μM), endplate potentials (e.p.ps) were recorded continuously from one endplate before and throughout application of toxin. Solution (10–20 ml) containing toxin at the desired concentration was perfused through the tissue bath for 30 min, with recycling of the solution after aeration; then toxin-free solution was used to perfuse the tissue. The preparations were maintained at 30°C. One preparation was used for each condition.

Presynaptic waveforms were recorded by glass microelectrodes (containing 2 M NaCl, resistance 5–15 MΩ) placed inside the perineural sheath (near endplate areas) of a branch of an intercostal nerve. The waveforms were displayed and recorded as for intracellular recording. Two preparations were used for each experimental condition, and values quoted are means ± s.e.mean.

### *Studies on spontaneous release during exposure to KCl and A23187*

Triangularis sterni preparations were used as described above, except that the temperature of the perfusing solution was 34°C. Miniature endplate potentials (m.e.p.ps) were recorded before and after addition of 10 mM KCl or 20 μM A23187 for 2 min. The preparations were then stimulated at 0.5 Hz and 140 nM β-bungarotoxin was added. After the muscle stopped twitching, a fibre was impaled with a microelectrode and recordings of e.p.ps and m.e.p.ps were made. Recordings of m.e.p.ps were then made after addition of 10 mM KCl or 20 μM A23187. As the effects of A23187 were difficult to reverse, separate preparations were used for control and β-bungarotoxin-treated samples. Three preparations were used for each treatment.

### *Materials*

Crotoxin and β-bungarotoxin were gifts from Dr C. Bon, Institut Pasteur, Paris, and Dr E. Karlsson, University of

Uppsala, respectively. Some β-bungarotoxin was also obtained from Calbiochem. A23187 (Calbiochem) was prepared as a stock solution of 2 mg ml$^{-1}$ in 95% ethanol. Other chemicals were obtained from Sigma Chemical Co., Poole, Dorset.

## Results

### *Effects on evoked release and nerve terminal action potentials*

In mammalian nerve-muscle preparations, the early facilitatory phase produced by β-bungarotoxin and crotoxin is apparently independent of the toxins' phospholipase activity. Hence, our previous experiments (Rowan & Harvey, 1988) were performed at room temperature to reduce enzymatic activity. To examine the blocking effects of the toxins, experiments were performed at 30°C and in the presence of normal concentrations of $Ca^{2+}$.

β-Bungarotoxin (150 nM) and crotoxin (130 nM) reduced the amplitude of e.p.ps in a time-dependent manner (Figure 1A and B). There was little change in the membrane potentials of the cells: for example, membrane potential before addition of β-bungarotoxin was −80 mV; after 60 min, it was −84 mV. At 30°C, there was no evidence for a facilitation of e.p.ps, probably because high concentrations of toxins were used and the blocking activity overwhelmed the facilitatory effect.

The perineural waveform consists of a small positive component followed by two larger negative components, which correspond to $Na^+$ and $K^+$ currents (see Rowan & Harvey, 1988 and Anderson *et al.*, 1988). In two control experiments where the temperature was raised to 30°C, the perineural waveform was stable for over 60 min. The waveform was markedly faster than at room temperature, but it showed the same sensitivity to ion channel blocking compounds as at room temperature. Thus, 200 μM 3,4-diaminopyridine abolished the second negative waveform and revealed a delayed positive component. Tetraethylammonium (1 mM) increased both amplitude and time course of the positive component, which was reduced by the $Ca^{2+}$ channel blocker $Cd^{2+}$ (200 μM).

When β-bungarotoxin (150 nM) or crotoxin (130 nM) was added to preparations maintained at 30°C, there was a selective reduction in the second negative deflection (Figure 1c and d). This occurred 5–10 min after exposure to the toxin, and the reduction, which was 31 ± 6% with β-bungarotoxin and 53 ± 2% with crotoxin, was stable for at least 60 min. The first negative component of the waveform did not change significantly from control. After exposure to toxin, 3,4-diaminopyridine still abolished the remainder of the second negative waveform and revealed a positive component that was enhanced by subsequent addition of tetraethylammonium. This positive component was similar to that seen in control preparations.

### *Effects of KCl and A23187*

M.e.p.p. frequency in control preparations was approximately doubled during 2 min exposure to 10 mM KCl or 20 μM A23187 (Figure 2). Preparations were then exposed to 140 nM β-bungarotoxin. Visible twitch responses to motor nerve stimulation could not be observed after 75–90 min. The m.e.p.p. frequency recorded after abolition of twitching was apparently normal (i.e. 1–4 Hz). M.e.p.ps could be recorded for at least 2.5 h after addition of the toxin; during this late phase, m.e.p.ps often occurred in high frequency bursts, as described by Abe *et al.* (1976) and large amplitude m.e.p.ps occurred more frequently.

After the muscle fibres stopped twitching, e.p.ps could be recorded for a short period before they were abolished. Quantal contents during this period were around 4–9. Shortly

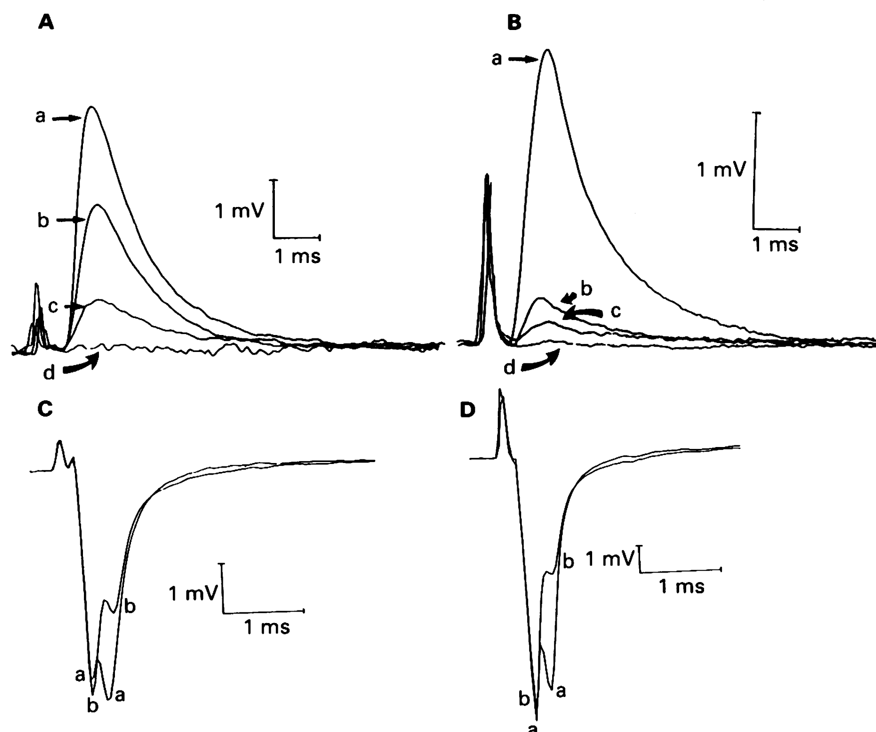


**Figure 1** Effect of β-bungarotoxin and crotoxin on nerve-evoked end plate potentials (e.p.ps) and perineural waveforms recorded from mouse triangularis sterni preparations at 30°C in the presence of 2.5 mM $Ca^{2+}$. (A) (a) Averaged control e.p.p.; (b–d) averaged e.p.ps from the same neuromuscular junction 5, 10, and 60 min after addition of β-bungarotoxin (150 nM). (B) Averaged control e.p.p.; (b–d) averaged e.p.ps from the same neuromuscular junction 5, 20, and 30 min after addition of crotoxin (130 nM). (C) (a) Averaged control waveform; (b) averaged waveform after 60 min exposure of β-bungarotoxin (150 nM). (D) (a) Averaged control. waveform; (b) averaged waveform after 60 min exposure of crotoxin (130 nM). Note the large decrease in the second component of the waveforms in the presence of the toxins. Calibration bars: 1 ms and 1 mV.

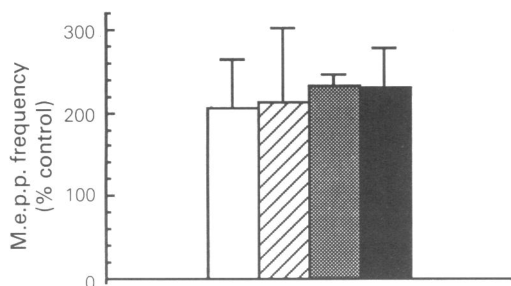


**Figure 2** Increase of miniature endplate potential (m.e.p.p.) frequency by KCl and A23187. Left-hand columns, responses to 10 mM KCl: open column, control; hatched column, after twitch blockade by 130 nM β-bungarotoxin. Right-hand columns, responses to 20 μM A23187: stippled column, control; solid column, after twitch blockade by 140 nM β-bungarotoxin. Frequencies in the absence of stimulation were taken as 100%. Each column represents the mean of three separate experiments; bars indicate s.e.means.

after twitch responses were blocked, preparations were exposed to 10 mM KCl or 20 μM A23187. Both treatments increased the frequency of m.e.p.ps to about the same extent as in non-toxin treated preparations (Figure 2). Once the frequency of m.e.p.ps had dropped to zero (about 150 min after addition of β-bungarotoxin), neither KCl nor A23187 restored m.e.p.ps.

## Discussion

β-Bungarotoxin and crotoxin abolished e.p.ps with little alteration in the perineural recordings made from terminal regions. The changes that were seen in the nerve terminal action potential were similar to those seen at room temperature, which are associated with a block of some presynaptic $K^+$ channels (Rowan & Harvey, 1988). Thus, alterations in nerve terminal action potentials cannot account for the block of transmitter release. Nerve terminal action potentials and $Ca^{2+}$ currents (as revealed by application of 3,4-diaminopyridine and tetraethylammonium) were still active even after acetylcholine release had been abolished. Previously, β-bungarotoxin (Chang *et al.*, 1973) and crotoxin (Breithaupt, 1976) were reported not to block conduction of action potentials in rat or rabbit phrenic nerves, respectively. Additionally, extracellularly recorded nerve terminal spikes in frog sartorius nerve-muscle preparations were unaffected by paralysis by β-bungarotoxin (Chang *et al.*, 1973). Chang *et al.* (1973) and Hawgood & Smith (1977) provided further evidence that action potentials could still invade nerve terminals of rat and mouse diaphragm preparations paralysed by β-bungarotoxin or crotoxin, respectively, because tetanic stimulation continued to increase the frequency of m.e.p.ps after failure of e.p.ps. This implies that the blockade of transmitter release must occur at some site of critical importance for the release mechanism but after the action potential.

We attempted to test the functioning of $Ca^{2+}$-dependent processes by directly depolarizing the nerve terminal with elevated extracellular $K^+$, and by introducing $Ca^{2+}$ ions via an ionophore in order to bypass the physiological $Ca^{2+}$ channels. After β-bungarotoxin had induced muscle paralysis, evoked acetylcholine release was severely impaired: quantal contents were usually 4–5, while in normal mouse neuromuscular junctions values of at least 30 are expected (Hong & Chang, 1989).

However, both $K^+$-induced depolarization and the $Ca^{2+}$ ionophore could still enhance the frequency of m.e.p.ps, as in control preparations. A brief report of apparently similar findings with the notexin homologue notechis II-5 was published by Kamenskaya & Satybaldina (1979). After prolonged exposures to β-bungarotoxin, KCl and A23187 had no effect, as previously reported for $K^+$ during β-bungarotoxin- (Oberg & Kelly, 1976) and crotoxin-induced paralysis (Chang & Lee, 1977).

Our results imply that when β-bungarotoxin has prevented evoked acetylcholine release, the nerve terminal still has functional release sites with apparently normal sensitivity to intracellular $Ca^{2+}$. The terminals are presumably not continuously depolarized because the perineural recordings are normal, m.e.p.p. frequency is not elevated, and the terminals respond normally to 10 mM KCl. The question remains: what is the site of action of β-bungarotoxin? One possibility is that the apparently normal functioning of the terminal $Ca^{2+}$ channels is misleading. Prolonged depolarization, as induced by KCl or in the presence of 3,4-diaminopyridine and tetraethylammonium, may open $Ca^{2+}$ channels additional to those involved in the physiological regulation of acetylcholine release. For example, acetylcholine release or the perineural waveform is not usually affected by $Ca^{2+}$-channel blockers such as verapamil, but verapamil-sensitive $Ca^{2+}$ currents are revealed during prolonged depolarization (Penner & Dreyer, 1986; Anderson & Harvey, 1987). Also, $Ca^{2+}$ channels differing in their sensitivity to depolarization have been characterized in other neurones (Nowycky *et al.*, 1985). Hence, the physiologically important $Ca^{2+}$ current may be obscured during a $K^+$-induced depolarization, and it could be blocked specifically by β-bungarotoxin and similar toxins.

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